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AMP-activated protein kinase is involved in perfluorohexanesulfonate -induced apoptosis of neuronal cells

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HIGHLIGHTS

- Ca^{2+} influx via NMDA receptor and L-type calcium channel is involved in PFHxS-induced apoptosis of PC12 cells.
- PFHxS-induced apoptosis of PC12 cells is AMPK- and ERK- dependent.
- AMPK and ERK are downstream molecules of increased intracellular Ca^{2+}.
- AMPK and ERK activation are regulated by distinct pathways.

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ABSTRACT

Perfluorohexanesulfonate (PFHxS), one of the major perfluoroalkyl compounds (PFCs), has been used in a variety of industrial and consumer applications and detected in serum in the general population. This raised a concern over its possible detrimental health effects, including neurotoxic effects. We have previously shown that PFHxS induced neuronal apoptosis via the NMDA receptor-mediated extracellular signal-regulated kinase (ERK) pathway. Recently, it has been reported that AMP-activated protein kinase (AMPK) acts as a key signal molecule in neuronal excitotoxicity as well as providing a neuroprotective function. In the present study, we have examined the involvement of AMPK in PFHxS-induced neuronal apoptosis using neuronal differentiated PC12 cells. PFHxS induced significant increases in intracellular [Ca^{2+}] via the NMDA receptor and the L-type voltage-gated calcium channel (L-VGCC). The inhibition of Ca^{2+} loading by the NMDA receptor antagonist, MK801 and the L-VGCC blockers, nifedipine and diltiazem significantly reduced PFHxS-induced apoptosis. PFHxS induced sustained activation of AMPK and the inhibition of AMPK activation by compound C and AMPK siRNA significantly reduced PFHxS-induced caspase-3 activity. These results indicate the pro-apoptotic role of AMPK. The activation of AMPK was attenuated by MK801, nifedipine and diltiazem. However, the activation of AMPK was not affected by the ERK inhibitor, PD98059. Likewise, ERK activation was not affected by compound C but was substantially reduced by MK801, nifedipine or diltiazem. This suggests that the activation of AMPK and ERK is regulated by intracellular Ca^{2+} loading in distinct pathways. Taken together, PFHxS-induced neuronal apoptosis is mediated by AMPK and ERK pathways, which are distinctly regulated by increased intracellular Ca^{2+} via the NMDA receptor and L-VGCC.

1. Introduction

Perfluoroalkyl compounds (PFCs) have been widely used in a variety of industrial processes and consumer products (Buck et al., 2011). Since PFCs are very stable and extremely persistent to degradation, PFCs are ubiquitous environmental contaminants and accumulate in the human body via exposure from the food web, drinking water and inhalation of dust (Gebbink et al., 2015; Vestergren et al., 2012). Perfluorohexanesulfonate (PFHxS), one of the major PFCs, has been detected in serum samples taken from the general population as well as in umbilical cord and breast milk (Kärrman et al., 2007; Kato et al., 2011; Lee et al., 2013). More importantly, the serum level of PFHxS in children has been reported to be greater than adults (Calafat et al., 2007; Olsen et al., 2004; Toms et al., 2009). This raised a concern over their health effects,

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including its neurotoxic effects. A recent animal study has shown that a single neonatal exposure to PFHxS caused behavioral and cognitive disturbance in adult mice (Viberg et al., 2013), suggesting neuronal toxic effects. We have also previously shown that PFHxS induced neuronal cell apoptosis (Lee et al., 2014). However, studies on its neuronal effects are limited and not much is known about the underlying mechanisms.

N-methyl-D-aspartic acid (NMDA) receptor, an ionotropic glutamate receptor (iGluR), mediates excitatory neural transmission. The activation of the NMDA receptor increases Ca^{2+} influx into the postsynaptic cells, which plays a critical role in synaptic plasticity, memory formation and learning (Crowder et al., 1987). In addition to its function of neural survival under physiological condition, the overstimulation of NMDA receptor induces excessive Ca^{2+} influx into the cells which results in neuronal cell damage known as excitotoxicity (Manev et al., 1989; Urushitani et al., 2001). The excitotoxic stimulation of the NMDA receptor has been associated with seizure, ischemia and neurodegenerative diseases (Auzmendi et al., 2009; Choi, 1994; Hsieh et al., 2012; Mota et al., 2014; Olivares et al., 2012). Therefore, the NMDA receptor has drawn more attention among GluR as a therapeutic target to treat many neuropathological conditions. We have previously reported that PFHxS-induced neuronal apoptosis was inhibited by the NMDA receptor antagonist (Lee et al., 2014), suggesting that PFHxS induces excitotoxic neuronal cell death.

The excess intracellular Ca^{2+} loading disturbs ion homeostasis and leads to decreasing the level of intracellular ATP. AMP-activated protein kinase (AMPK) is activated in response to ATP depletion and is implicated in a series of catabolic pathways to restore cellular energy level, exerting its neuroprotective effect (Weisová et al., 2012). In addition to its involvement in metabolic processes, AMPK plays an important role in neuronal apoptosis (Garcia-Gil et al., 2003; Concannon et al., 2010). This suggests that AMPK has dual functions on neuronal fate. However, the involvement of AMPK activation in PFHxS-induced neurotoxicity is not known. In the present study, we examined the role of AMPK in PFHxS-induced neuronal apoptosis and the involvement of the NMDA receptor in AMPK activation, using the neuronal differentiated rat pheochromocytoma cell line, PC12 cells.

2. Materials and methods

2.1. PC12 cell culture

PC12 cells were purchased from the Korean cell line bank and maintained in RPMI 1640 Medium supplemented with 10% horse serum (HS), 5% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37 °C in a humidified atmosphere of 5% CO_2. The cells at passage 18–30 were used for experiments. For neuronal differentiation, cells were seeded at $1.4 \times 10^4/cm^2$ on poly-L-lysine coated dishes. Cells were allowed to attach overnight and were differentiated by replacement with RPMI 1640 containing 1% HS, 5% FBS, 1% P/S and 100 ng/ml nerve growth factor (NGF) for five days. The differentiation medium was changed every two days. The differentiated PC12 cells were analyzed by detecting neurite formation under the phase-contrast microscope (U-LH 100-3, Olympus) and GAP-43 expression by western blotting.

2.2. Intracellular $[Ca^{2+}]$ measurement

The intracellular $[Ca^{2+}]$ ($[Ca^{2+}]_i$) was measured using a calcium detection kit according to the manufacturer's instructions (Abcam, Cambridge, MA, USA). Briefly, cells grown on 60 mm dishes were homogenized and centrifuged at 15,000 rpm for 15 min at 4 °C. The supernatant was collected and reacted with a chromogenic reagent, 0-cresolphthalein (Goonasekera SA et al., 2014; Stern and Lewis, 1957). The absorbance of formed chromophore was measured at 575 nm.

2.3. Caspase-3 activity measurement

The caspase-3 activity was measured using a caspase-Glo kit according to the manufacturer's instructions (Promega, Madison, WI). Briefly, cells grown on 96- well plates (3×10^4 cells/100 μl// well) were treated with luminogenic substrate containing the DEVD sequence (Bayascas et al., 2002). The cleaved caspase substrate generates a luminescent signal and the relative light units were measured using a Plus LB 96 V luminometer (Berthold Detection System, Oak Ridge, TN). The intensity of luminescence is proportional to the amounts of caspase activity.

2.4. Western blotting

Western blot analysis was performed as described previously (Lee et al., 2014). Equal amounts of protein from whole cell extracts were separated by SDS-PAGE gel and transferred to a nitrocellulose membrane. After blocking the membrane with 5% non-fat dry milk, the blots were incubated with anti-phospho-AMPK, anti-AMPK, anti-phospho-ACC, anti-ACC, anti-phospho-ERK, anti-ERK antibodies (Cell signaling, Beverly, MA), anti-GAP-43 antibody (Invitrogen, Carlsbad, CA) and anti-GAPDH antibody (Santa Cruz, Dallas, TX), and then reacted with a peroxidase-conjugated secondary antibody. The protein bands were detected by Super Signal (Pierce, Rockford, IL). The density of respective bands was analyzed by the Chemi-Doc XRS imaging system (Bio-Rad, Hercules, CA). The membranes were reprobed with anti-GAPDH antibody, which was used as the loading control.

2.5. TUNEL assay

The TUNEL assay (Gavrieli et al., 1992) was performed by using a terminal deoxynucleotidyl transferase (TdT)-mediated dUTP-nick end-labeling (TUNEL) assay kit (DeadEnd TM fluorometric TUNEL system; Promega, Madison, WI) according to the manufacturer's protocol. TUNEL-positive cells were analyzed under a fluorescence microscope (U-LH 100-3, Olympus).

2.6. Transfection with small-interfering RNA (siRNA)

Differentiated PC12 cells were transfected with 100 nM AMPK siRNA (50 nM α1 and 50 nM α2) (Santa Cruz, Dallas, TX) or 100 nM non-targeting scrambled siRNA (Ambion, Carlsbad, CA) using lipofectamin RNAiMAX in OPTI-MEM (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. After 48 h of transfection, cells were harvested for further experiments.

2.7. Statistics

Data are expressed as mean ± SEM. Statistical analyses were made by the Student's *t*-test to compare values between two groups or by one way ANOVA followed by Tukey's post hoc test to compare values among more than three groups using GraphPad Prism (version 4.0). A value of $P < 0.05$ was considered significant.

3. Results

3.1. The neuronal differentiation of PC12 cells

The neurite formation of cells by NGF treatment was observed under a microscope to analyze neuronal differentiation of

PC12 cells. Cells were maintained in differentiation medium containing 100 ng/ml of NGF. After 24 h of NGF treatment (day 1), neurite-forming cells were noticeable. The number of PC12 cells with neurites and the length of neurites increased with time. On day 5, more than 90% of cells generated neurites (Fig. 1A). The neuronal differentiation of PC12 cells was further examined by detecting growth associated protein 43 (GAP-43), a neuronal marker. The protein level of GAP-43, barely detectable in undifferentiated PC12 cells (control), was increased by NGF treatment (Fig. 1B). Therefore, cells were treated with NGF for 5 days for neuronal differentiation in subsequent experiments.

3.2. The involvement of Ca^{2+} influx via the NMDA receptor and the L-type voltage-gated calcium channel in PFHxS-induced apoptosis of PC12 cells

In a previous study, we have shown that the increased caspase-3 activity induced by PFHxS was reduced by the NMDA receptor antagonist, MK801 (Lee et al., 2014). To determine the involvement of NMDA receptor activation in PFHxS-induced apoptosis, concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) was measured. PFHxS induced a significant increase in the level of $[Ca^{2+}]_i$ at 5 min, which progressively increased to a maximum increase at 1 h and gradually returned to basal at 24 h (Fig. 2A). The elevation of $[Ca^{2+}]_i$ by PFHxS was almost completely blocked by MK801 and the L-type voltage-gated calcium channel (L-VGCC) blockers, nifedipine and diltiazem (Fig. 2B). These results indicated that PFHxS induced persistent Ca^{2+} influx which occurs primarily via the NMDA receptor and L-VGCC.

In an attempt to determine the role of $[Ca^{2+}]_i$ influx by PFHxS, caspase-3 activity was measured. As reported previously, MK801 significantly reduced casaspe-3 activity. Nifedipine and diltiazem also attenuated the increased caspase-3 activity by PFHxS with an extent similar to MK801 (Fig. 2C). This result suggests that PFHxS induced Ca^{2+} influx is involved in neuronal cell apoptosis.

3.3. The involvement of AMPK in PFHxS-induced apoptosis of PC12 cells

The effect of PFHxS on AMPK activation was examined by Western blotting. Cells were treated with 300 μM PFHxS for different time points (0–24 h). The phosphorylation of AMPK significantly increased at 6 h and the increase remained up to 24 h (Fig. 3A and B), showing persistent activation of AMPK by PFHxS.

To examine the role of AMPK in PFHxS-induced apoptosis, caspase-3 activity was measured in the presence of compound C, an AMPK inhibitor. Compound C inhibited the phosphorylation of AMPK (Fig. 3C) and significantly reduced caspase-3 activity induced by PFHxS (Fig. 2C). The TUNEL assay showed consistent results (Fig. 3D). This indicated that AMPK plays a pro-apoptotic role in PFHxS-induced apoptosis. This result was further confirmed by using AMPK siRNA. Cells were treated with AMPK siRNA or scramble siRNA. As expected, AMPK siRNA significantly reduced PFHxS-induced AMPK activation and AMPK protein level whereas scramble siRNA had no effect (Fig. 3E). Consequently, the phosphorylation of acetyl-CoA carboxylase (ACC), a downstream target of AMPK, was substantially reduced by AMPK siRNA without changes in its protein level. Similar to the effect of compound C, AMPK siRNA significantly reduced PFHxS-induced caspase-3 activation (Fig. 3F).

3.4. The regulation of AMPK and ERK activation by PFHxS in PC12 cells

The role of $[Ca^{2+}]_i$ in PFHxS-induced AMPK activation was investigated. The phosphorylation of AMPK and ACC by PFHxS was attenuated by MK801, nifedipine and diltiazem (Fig. 4A), indicating that the activation of AMPK is regulated by increased intracellular Ca^{2+}. However, compound C had no effect on PFHxS-induced Ca^{2+} influx (Fig. 2B). This suggests that intracellular Ca^{2+} is an upstream signaling molecule of AMPK activation but not vice versa.

In a previous study, we have shown that ERK is pro-apoptotic and is one of the downstream molecules of the NMDA receptor pathway. Therefore, the crosstalk between AMPK and ERK pathways was examined. Similar to AMPK activation, ERK activation was decreased by MK801, nifedipine and diltiazem but not by compound C (Fig. 4B). Likewise, AMPK activation was not changed by PD98059, an inhibitor of the ERK pathway (Fig. 4A). Compared to the treatment with either compound C or PD98059 alone, the co-treatment of cells with compound C and PD98059 further reduced caspase-3 activity, albeit not significantly (Fig. 4C). These

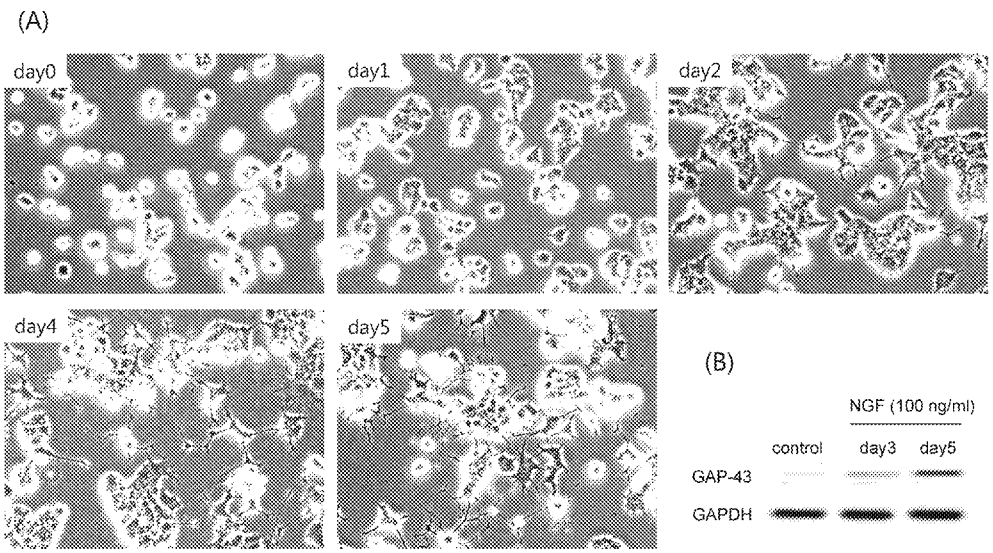

Fig. 1. The neuronal differentiation of PC12 cells. Cells were treated with 100 ng/ml of NGF for 5 days. (A) The formation of neurite was observed under the phase-contrast microscope (U-LH 100-3, Olympus). Representative microscopic images from three independent experiments were presented (magnification, x 200). (B) The expression of GAP-43 was detected by Western blotting.

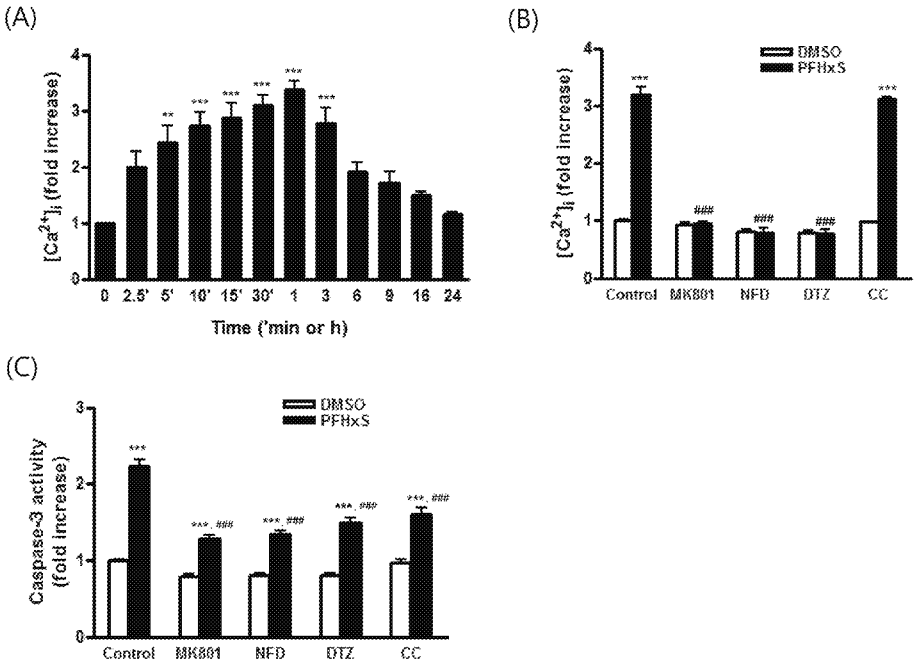

Fig. 2. The involvement of Ca^{2+} influx in PFHxS-induced apoptosis of PC12 cells. (A) Cells were treated with 300 μM of PFHxS for different time points (0–24 h). The level of $[Ca^{2+}]_i$ was measured. Cells were pretreated with MK801 (1 μM), NFD (10 μM), DTZ (10 μM) or CC (5 μM) and then stimulated with 300 μM of PFHxS for 1 h to detect $[Ca^{2+}]_i$ (B) and for 24 h to measure caspase-3 activity (C), respectively. Data (fold increase) are mean ± SEM of three independent experiments. $^{**}P < 0.01$, $^{***}P < 0.001$ vs. DMSO. ###$P < 0.001$ vs. corresponding Control cells (NFD, nifedipine; DTZ, diltiazem; CC, compound C).

results indicated that both AMPK and ERK are downstream signal molecules following increased intracellular Ca^{2+}, but each pathway is regulated differently.

4. Discussion

Environmental pollution is considered one of the important etiologies of neurodegenerative diseases. Increasing number of studies suggest that neuronal exposure to environmental toxicants in early life increases the risk of neurodegenerative diseases (Fox et al., 2012). PFHxS, one of the major PFCs, is a ubiquitous environmental contaminant and has been detected in the general population with higher concentrations in children than what has been observed in adults. Several studies have suggested it is a possible neurotoxin. However, the underlying mechanism responsible for its potential neuronal effects is largely unknown.

In the present study, we have shown that PFHxS induced neuronal apoptosis in an AMPK-dependent manner, where increased intracellular Ca^{2+} via the NMDA receptor and L-VGCC is one of the upstream signal molecules of AMPK activation. Calcium ion plays a critical role in numerous cellular functions and its intracellular concentration is finely regulated under physiological conditions. Overactivation of the NMDA receptor, an ionotropic glutamate receptor (iGluR), results in excess Ca^{2+} influx, which causes disturbance of intracellular ion homeostasis leading to impairment of neuronal function and neuronal death (Manev et al., 1989; Urushitani et al., 2001). This excitotoxic neuronal damage is known to be a critical event implicated in numerous neuropathological conditions including ischemia, seizure and neurodegenerative diseases (Auzmendi et al., 2009; Choi, 1994; Hsieh et al., 2012; Mota et al., 2014; Olivares et al., 2012). PFHxS induced a persistent increase in $[Ca^{2+}]_i$ and the inhibition of Ca^{2+} influx by an NMDA receptor antagonist significantly reduced PFHxS-induced neuronal apoptosis. This indicated that PFHxS induced an NMDA receptor-mediated excitotoxic neuronal death. Although Ca^{2+}-mediated neuronal excitotoxicity has been considered to occur mainly through NMDA receptor activation (Aarts et al., 2002), Ca^{2+} entry in neuron also occurs via several different routes, including L-VGCC and Na^+/Ca^{2+} exchanger (Berman and Murray, 2000). A massive Ca^{2+} influx and membrane depolarization by overactivation of the NMDA receptor cause L-VGCC activation and, in turn, further increase $[Ca^{2+}]_i$ by Ca^{2+}-induced Ca^{2+} release from intracellular calcium storage (Fagni et al., 2000; Sukhareva et al., 2002). In the present study, L-VGCC blockers completely blocked PFHxS-induced intracellular Ca^{2+} loading and subsequently inhibited caspase-3 activation. This suggests that L-VGCC also contributes to PFHxS-induced excitotoxic neuronal apoptosis. Previous studies have supported the contribution of L-VGCC to neuronal excitotoxicity. The vulnerability of hippocampal neurons to stimulus has been shown to increase with age in proportion to L-VGCC expression level (Brewer et al., 2007; Stanika et al., 2009). Therefore, the contribution of L-VGCC in neuronal excitotoxicity can differ depending on the type of stimuli and the experimental conditions. In the present study, the increased $[Ca^{2+}]_i$ by treatment of cells with PFHxS for 1 h was almost completely blocked by both an NMDA receptor antagonist and L-VGCC blockers. Because L-VGCC activation is dependent on NMDA receptor-induced membrane depolarization (Magee and Johnston, 1995), it is speculated that both the NMDA receptor and L-VGCC played a role in Ca^{2+} entry at this time point. Further study on elaborating the sources of increased intracellular Ca^{2+} loading by PFHxS is warranted.

AMPK, a serine/threonine kinase composed of heterotrimeric (α/β/γ) subunits, is activated in response to ATP depletion with increased AMP/ATP ratio and is involved in various cellular metabolic processes to restore cellular energy level. AMPK is highly expressed in neurons and the activation of AMPK induced by metabolic or other toxic insults has been shown to be neuroprotective (Poels et al., 2009; Weisová et al., 2009, 2012). However, AMPK activation also mediates neuronal apoptosis and the inhibition of AMPK protects neurons from the neurotoxic effects of

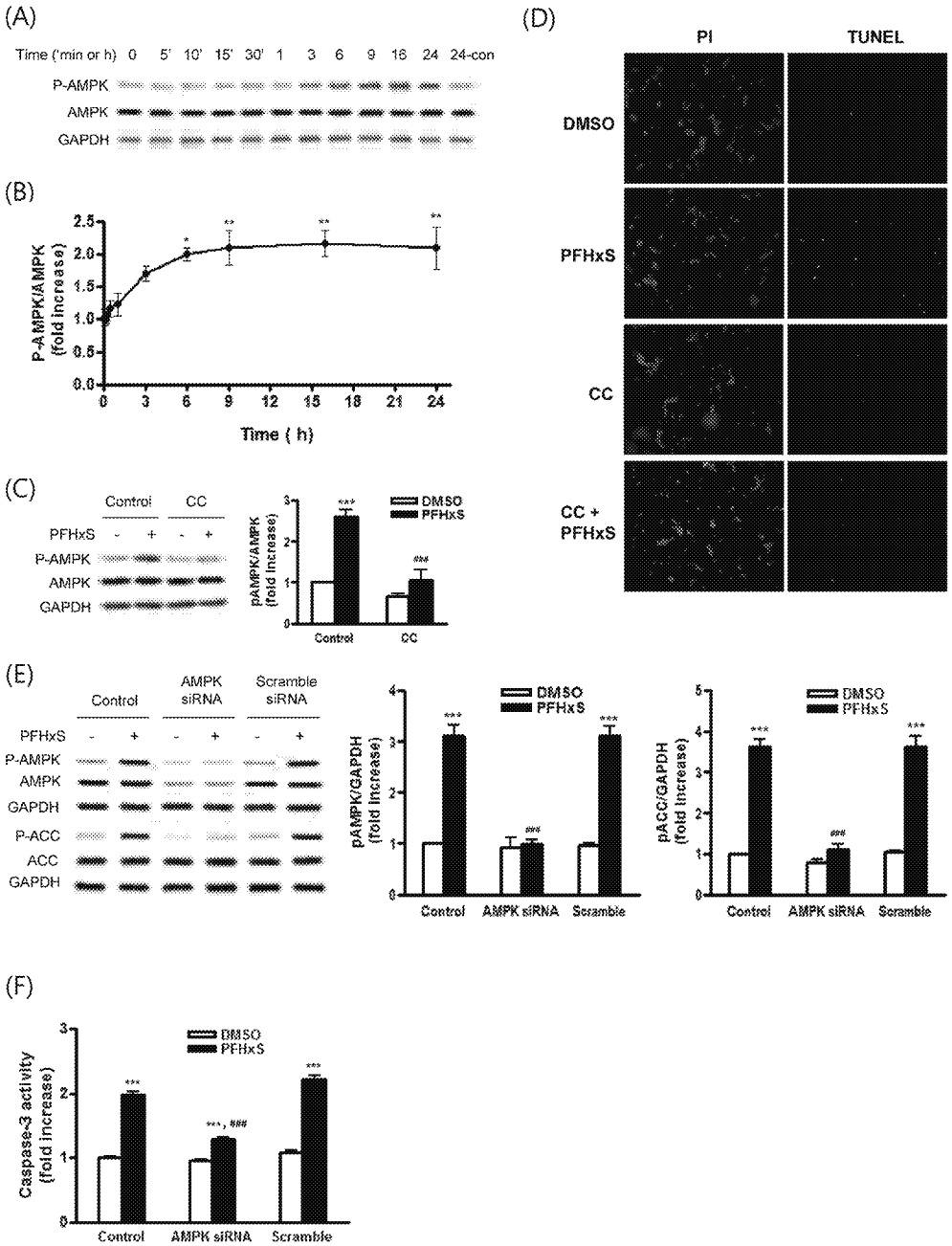

Fig. 3. The role of AMPK in PFHxS-induced apoptosis of PC12 cells. (A, B) Cells were treated with 300 μM of PFHxS for different time points (0–24 h). The levels of phosphorylated- and total protein of AMPK were detected by Western blotting. The blots are representative of three independent experiments. The densities of bands from Western blotting were measured and the fold increases in ratio P-AMPK/AMPK were presented. (C) Cells were pretreated with 5 μM of CC for 3 h and then stimulated with 300 μM of PFHxS for 6 h to detect the level of phosphorylated AMPK by Western blotting. The densities of bands were measured and the fold increase in ratio pAMPK/AMPK was presented. (D) Cells were treated with DMSO control or PFHxS (300 μM) in the presence or absence of compound C (5 μM) for 24 h. The TUNEL positive cells were monitored by fluorescence microscopy after staining cells with PI and TUNEL staining. Representative microscopic images from three independent experiments were presented (magnification, x 200). (E, F) Cells were transfected with AMPK siRNA, scramble siRNA for 48 h and then treated with 300 μM of PFHxS for 6 h and 24 h to detect the level of phosphorylated AMPK and caspase-3 activity, respectively. The densities of bands were measured and the fold increases in ratio pAMPK/GAPDH and pACC/GAPDH were presented. Data (fold increase) are mean ± SEM of three independent experiments. . $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. DMSO. ###P < 0.001 vs. corresponding Control cells (CC, compound C).

energy depletion, amyloid-β oligomer and stroke (Mairet-Coello et al., 2013; Concannon et al., 2010; McCullough et al., 2005), suggesting the dual effects of AMPK in neuronal survival and death. It has been suggested that the kinetics of AMPK activation is an important determining factor for its cellular responses. The transient activation of AMPK after excitotoxic stimuli has been shown to be involved in neuroprotection by increasing glucose transporters 3 (GLUT3) expression at the plasma membrane (Weisova et al., 2009) whereas prolonged activation of AMPK has been shown to be involved in neuronal apoptosis by increasing the expression of Bim, a pro-apoptotic molecule (Concannon et al., 2010). In agreement with these observations, PFHxS induced a delayed and prolonged activation of AMPK which plays a pro-apoptotic role.

Overactivation of the glutamate receptor causes intracellular Ca^{2+} overloading which can lead to impaired mitochondrial bioenergetics and energy imbalance. Consequent ATP depletion

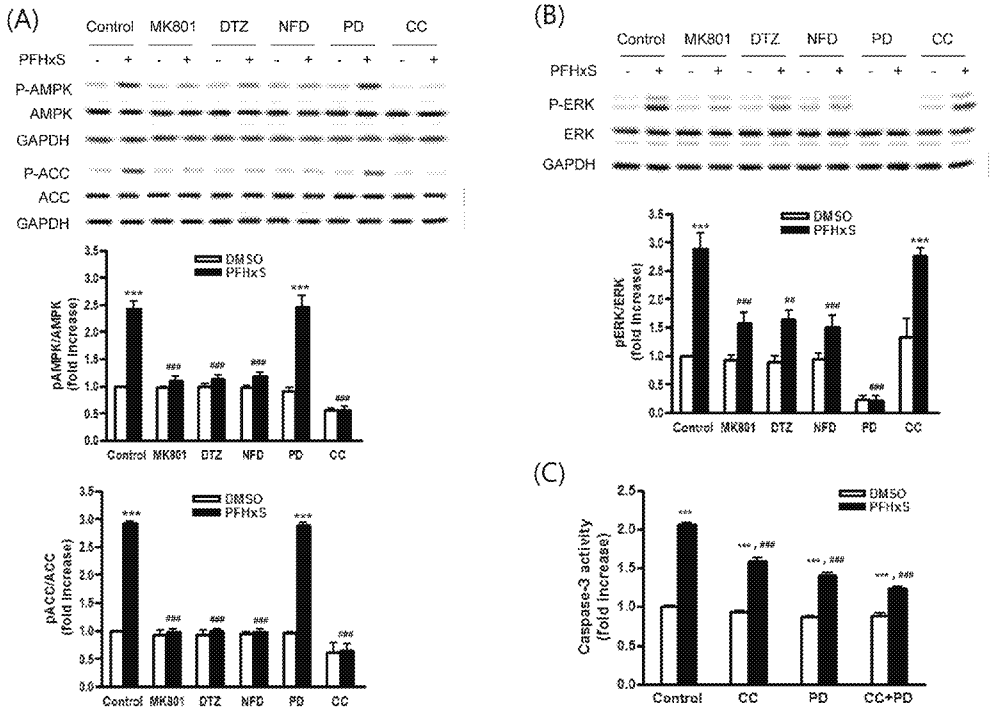

Fig. 4. The regulation of AMPK and ERK activation by PFHxS in PC12 cells. Cells were treated with 300 μM of PFHxS for 6 h (A) or 1 h (B) in the presence or absence of MK801 (1 μM), DTZ (10 μM), NFD (10 μM), PD98059 (50 μM) or CC (5 μM). Then, the phosphorylation of AMPK and ERK was detected by Western blotting. The densities of bands were measured and the fold increases in ratio pAMPK/AMPK, pACC/ACC and pERK/ERK were presented. (C) Cells were pretreated with CC (5 μM), PD (50 μM) or CC + PD (5 μM + 50 μM) and then stimulated with 300 μM of PFHxS for 24 h to detect caspase-3 activity. Data (fold increase) are mean ± SEM of three independent experiments. ***P < 0.001 vs. DMSO. ###P < 0.001 vs. corresponding Control cells (DTZ, diltiazem; NFD, nifedipine; CC, compound C; PD, PD98059).

activates AMPK, a cellular energy sensor (Suter et al., 2006). This suggests that increased $[Ca^{2+}]_i$ following NMDA receptor activation is one of the upstream molecules regulating AMPK activation in neurons. In line with previous studies, PFHxS-induced activation of AMPK was prevented by an NMDA receptor antagonist and L-VGCC blockers. In addition to allosteric activation by AMP binding, AMPK is activated by phosphorylation on Thr172 residue by upstream kinases including liver kinase B1 (LKB1) and Ca^{2+}/calmodulin-dependent kinase kinase 2 (CaMKK2). The activation of CaMKK2 is dependent on increased $[Ca^{2+}]_i$ (Hawley et al., 2005; Woods et al., 2005). This indicates that CaMKK2 may play an important role in AMPK activation during neuronal excitotoxicity. Therefore, identification of upstream kinase involved in AMPK activation as well as the downstream molecule in PFHxS-induced excitotoxicity is warranted in future studies.

In addition to the AMPK pathway, PFHxS-induced neuronal apoptosis has been shown to be dependent on the intracellular Ca^{2+}-mediated ERK pathway (Lee et al., 2014). Recent studies by others have shown that AMPK phosphorylation was positively regulated by ERK which was involved either in the inhibition of cell growth or the anti-apoptotic process (Tang et al., 2015; Zhang et al., 2015). Therefore, we have examined the crosstalk between AMPK and ERK. The phosphorylation of AMPK induced by PFHxS was not attenuated by ERK inhibition. Likewise, ERK phosphorylation was not affected by the AMPK pathway, indicating a lack of crosstalk between AMPK and ERK. Similar to our observation, Budinger et al. (2008) has shown that the activation of AMPK and ERK in rat epithelial alveolar type II cells is mediated by distinct pathways. Furthermore, AMPK and ERK pathways have been shown to be negatively regulated by each other (Damm et al., 2012; Kawashima et al., 2015; Cheng et al., 2011; Tillu et al., 2012). This suggests that the crosstalk between AMPK and ERK may be differentially regulated depending on the types of stimuli and cells.

In conclusion, AMPK and ERK play pro-apoptotic roles in neurons exposed to PFHxS, which are mediated by NMDA receptor and L-VGCC in a distinct manner. These findings provide some evidence for the underlying mechanisms responsible for PFHxS-induced neuronal damage, which may contribute to identifying target molecules for assessing PFC-related neurotoxicity.

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